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Subcuticular Rejection: an Advanced Mode of the Allogeneic Rejection in the Compound Ascidians *Botrylloides simodensis* and *B. fuscus*

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Abstract. Allogeneic rejection between colonies (colony specificity) was described by electron microscopy in two compound ascidians, *Botrylloides simodensis* and *B. fuscus*. When two incompatible colonies are brought into contact at their growing edges, the tunic cuticle dissolves and the tunics of the colonies partially fuse. Alloreactive, humoral factors may diffuse to the opposite colony through the partially fusing tunic, and the tunic cells (free cells distributed in the tunic) possibly recognize these factors and induce a rejection reaction. Then, blood cells—mainly morula cells—infiltrate into the tunic, while tunic cells are disintegrating near where the partial fusion of the tunic is occurring. The infiltrating blood cells aggregate, disintegrate, and discharge electron-dense materials in the tunic at the subcuticular regions where the tunics have partially fused. Since the rejection lesion is restricted to the subcuticular area, some regulatory systems may be involved in this restriction. At the end, new walls are formed in the tunic matrix to separate the rejection lesion from the contacting colonies. The new wall is a continuous layer composed of electron-dense fibers and is structurally identical to the regenerating tunic cuticle. The mode of occurrence of colony specificity (Hirose *et al.*, 1994) and the present results indicate that tunic cells are the only allorecognition sites in *B. fuscus*.

Introduction

Self or non-self recognition is one of the most fundamental interactions among individuals, and its occurrence has been observed in many animal phyla. For instance, rejection or acceptance of grafted tissues among allogeneic individuals (histocompatibility) is one of the immediate examples of its occurrence. Whereas a tissue graft is an artificial treatment, a kind of tissue transplantation occurs naturally in many colonial forms of sessile animals, from sponges to ascidians. This phenomenon is known as colony specificity; when a sessile colony grows on a substratum and comes in contact with another conspecific colony, the two colonies either fuse to form a single colony, or they do not fuse. The outcome depends on allogeneic recognition between the two colonies. In some ascidians, the genetic control of this allorecognition has been demonstrated and is thought to be an ancestor of the major histocompatibility complex in vertebrates (Oka and Watanabe, 1957, 1960; Sabbadin, 1962; Scofield *et al.*, 1982; Weissman *et al.*, 1990).

Studies on colony specificity in colonial ascidians have been focused on species of the family Botryllidae (botryllid ascidians), and every botryllid ascidian studied has exhibited colony specificity (reviewed in Saito *et al.*, 1994). These species all form sheetlike colonies in which the zooids are connected with a vascular network, and the whole of the colony is embedded in a gelatinous tunic, an integumentary matrix that covers the epidermis. Free cells (tunic cells) of a few different kinds are distributed throughout the tunic (Hirose *et al.*, 1991), and a cuticular layer overlaying the tunic matrix is the outermost structure of the colony (Hirose *et al.*, 1990a).

When two compatible colonies are brought into contact at their growing edges, they fuse into a single colony. The course of the fusion reaction is fundamentally the same in all botryllids, as follows: (1) contact of the tunic

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surfaces (= cuticle); (2) dissolution of the tunic cuticle at the contact area; (3) penetration of ampullae (termini of the blood vessels at colony periphery) into the opposite colony; (4) contact between the penetrating ampullae and those of the opposite colony; (5) ampullar fusion and establishment of vascular connection between the two colonies. In contrast, the rejection reaction in each species is initiated at a specific stage of the fusion process. In *Botryllus primigenus*, for example, rejection is initiated after the penetration of ampullae (Taneda, 1985).

Some *Botrylloides* species show a subcuticular rejection (SCR) that begins at the earliest step in the fusion process—immediately after the partial fusion of the tunic. The rejection reaction in SCR involves blood cell infiltration and occurs in only a small area of the tunic contacting an allogeneic colony. The loss of tissue is minimized in this rejection, which seems, therefore, to be the most advanced mode of rejection found thus far among botryllid ascidians. In other ascidians, such as *Botryllus primigenus*, all of the ampullae interacting with their allogeneic counterparts disintegrate and are cut off the colony (Taneda *et al.*, 1985, for review).

Among species of *Botrylloides* that show SCR, two different reactions are elicited when incompatible colonies are brought into contact at artificial cut surfaces. Whereas intensive rejection occurs at the contacting boundaries in *B. simodensis* (Hirose *et al.*, 1990b), vascular connections are established between incompatible conspecifics (surgical fusion) in *B. fuscus* and *B. violaceus* (Hirose *et al.*, 1988, 1994). Surgical fusion indicates that the effector system inducing the rejection in the latter two species is not distributed in the vascular system. During SCR, therefore, allorecognition is probably carried out in the tunic. We have used light and electron microscopy to examine the process of SCR in *B. simodensis* and *B. fuscus*. This study should provide a better understanding of the mechanisms of allogeneic rejection and bring essential insights to the questions: when is self or non-self recognized; and what is the agent of recognition?

Materials and Methods

Animals

Colonies of *Botrylloides simodensis* and *B. fuscus* were collected in the vicinity of Shimoda (Shizuoka Prefecture, Japan). The colonies were attached on glass plates and reared in culture boxes immersed in Nabeta Bay near the Shimoda Marine Research Center.

The fusibility of colonies was tested with fusion experiments, as follows: Two pieces of colony of the same size were brought into contact at their growing edges on a glass slide. After 1–2 h in a moisture chamber, the colonies attached to the glass slide and were then reared in running seawater in an aquarium in the laboratory; they were observed every day under a binocular stereomicroscope. Within several days, either a fusion or a rejection reaction occurred between the paired colonies. Here 'fusion' means establishment of a common vascular system between the two colonies, and 'rejection' means its interruption or prevention.

Light microscopy

The specimens were fixed with 2.5% glutaraldehyde solution containing 0.45 *M* sucrose buffered with 0.1 *M* cacodylate at pH 7.4. The fixed specimens were then dehydrated through a butanol series, embedded in Paraplast, sectioned at 5 μm, and stained with Congo red, Delafield's hematoxylin, and eosin-orange G.

Scanning electron microscopy

To observe the structures of internal tissues, the method of Armstrong (1971) was applied, as follows: Specimens embedded in Paraplast were cut with a razor blade or a microtome blade to expose the inner structures. The specimens were washed in xylene (1 h, three changes) to remove the Paraplast and then cleared with absolute ethanol. These specimens were dried in a critical point dryer and sputter-coated with gold-palladium. They were examined with a Hitachi S-570 scanning electron microscope at 20 kV.

Transmission electron microscopy

The specimens were fixed at room temperature in a 2.5% solution of glutaraldehyde containing 0.45 *M* sucrose and buffered with 0.1 *M* cacodylate at pH 7.4. An alternative fixation, on ice for 2 h, was in a 2.5% solution of glutaraldehyde containing 2% NaCl buffered with 0.1 *M* Millonig's phosphate buffer at pH 7.4. The latter medium is essentially the same as that of Sugino *et al.* (1987). These prefixed specimens were rinsed in the same buffer, and then postfixed with 1% osmium tetroxide in the same buffer for 1 to 2 h. After dehydration through an ethanol series, the specimens were cleared with *n*-butyl glycidyl ether and embedded in epoxy resin. Thin sections were double-stained with uranyl acetate and lead citrate. They were examined with a Hitachi HS-9 transmission electron microscope at 75 kV.

Results

General features of subcuticular rejection

In both *Botrylloides simodensis* and *B. fuscus*, when incompatible colonies are brought firmly into contact at their growing edges, they never fuse (Fig. 1). Yet the signs of inflammatory rejection are barely observable under a

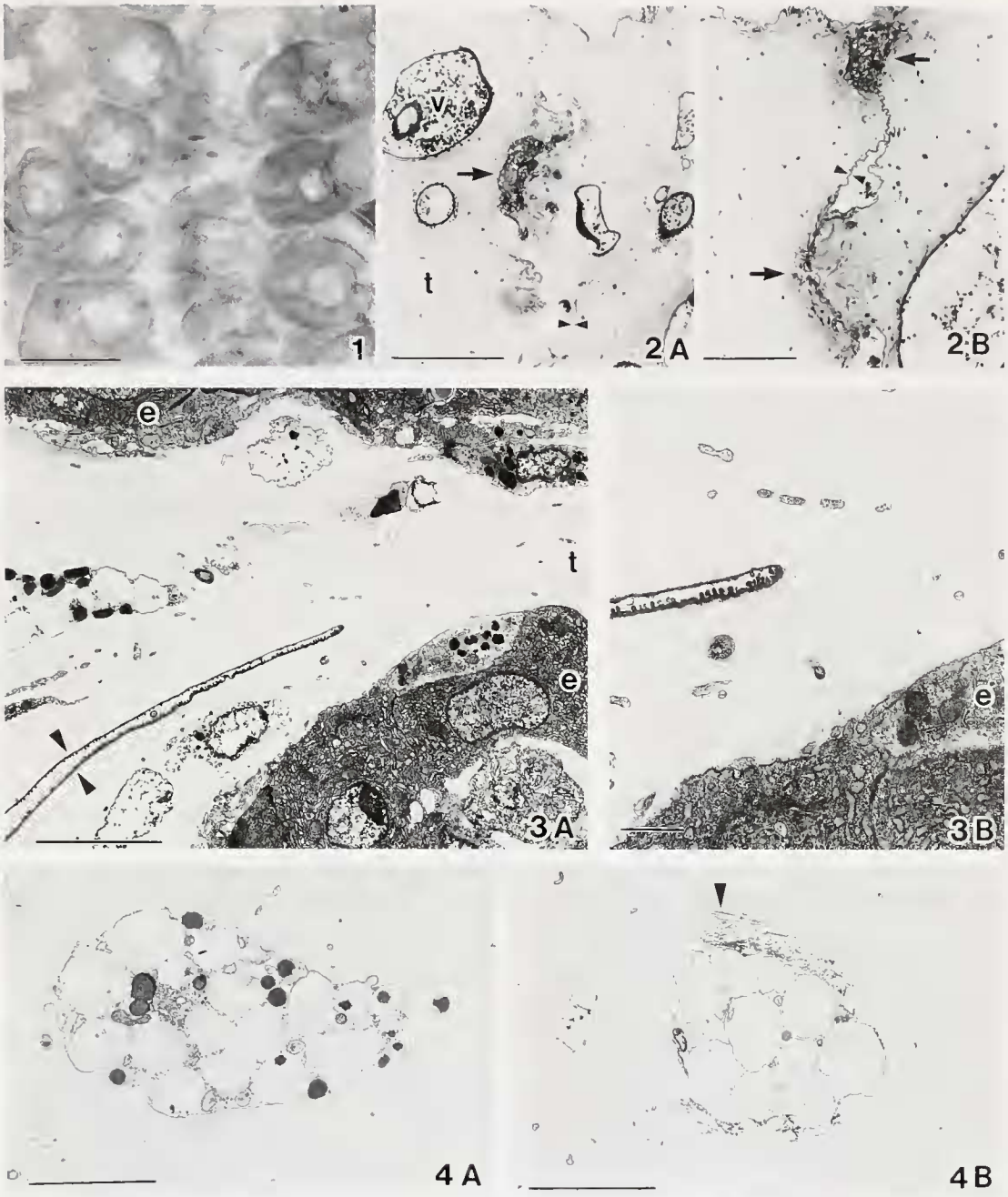


Figure 1. Subcuticular rejection in *Botrylloides simodensis*. Two incompatible colonies (right and left) are contacted at their growing edges. Rejection lesions are barely seen under the stereomicroscope. Bar = 0.5 mm.

Figure 2. Histological sections of subcuticular rejection in *Botrylloides simodensis* (A) and *B. fuscus* (B). Arrows, rejection lesions; arrowheads, tunic cuticle; t, tunic matrix; v, vascular vessel. Bar = 200 μm (A) and 100 μm (B).

Figure 3. Fusion of the tunic between incompatible colonies of *Botrylloides fuscus* (A) and an enlargement (B). Arrowheads, tunic cuticle of each colony; e, epidermis of vascular ampulla; t, tunic matrix. Bar = 5 μm (A), 1 μm (B).

Figure 4. Vacuo-granular tunic cells of *Botrylloides fuscus*. Intact cell with vacuoles and granules (A), and a disintegrating one discharging electron-dense materials that produce filamentous structures (arrowhead, B). Bar = 5 μm.

binocular stereomicroscope. In histological sections, rejection lesions are restricted to a limited part of the subcuticular region in the contact area (Fig. 2). In this rejection, infiltrating blood cells—mainly eosinophilic morula cells—aggregate and disintegrate in the tunic at the subcuticular region; thus the lesion is also well stained by eosin. The morphological process of SCR is fundamentally the same in *B. simodensis* and *B. fuscus*.

In this report, SCR is divided into the following three stages: (1) partial fusion of the tunic at the contact area; (2) blood cell infiltration, migration, and disintegration in the tunic; (3) separation of rejection lesion from the colonies. Since we cannot observe these processes in living specimens, the time course is difficult to define with precision. When actively growing colonies were brought into contact and reared at about 20°C, the time course could be estimated on the basis of histological data, as follows: (1) partial fusion of the tunic cuticle occurs 1 to 2 days after contact; (2) blood cell infiltration and disintegration begins shortly after partial fusion (2 to 3 days after contact) and continues until the rejection lesion is separated from the colony; (3) separation of the lesion is usually completed within 4 to 7 days after contact. The time course depends on the growth rate of the colonies as well as on temperature.

Partial fusion of the tunic

The first stage of SCR between the two colonies is the partial fusion of the contacting tunics; in other words, the dissolution of the tunic cuticles (Fig. 3). Partial fusion occurs in 1 to 2 days after the contact of the colonies. The tunic cuticle is a continuous, electron-dense sheet with minute protruberances (Hirose *et al.*, 1990a), and it is a boundary between the two contacting colonies. At the contact area, the tunic cuticles of both colonies adhere closely to each other. Thereafter, the cuticles dissolve here and there, and the tunic matrices of both colonies become continuous. In these areas, the edges of the adhering tunic cuticles become continuous and have a hairpin form (Fig. 3B). At this stage, the reaction between incompatible colonies shows no sign of a rejection reaction.

Blood cell infiltration, migration, and disintegration

The first signs of a rejection reaction are the infiltration of blood cells and the disintegration of tunic cells (vacuogranular tunic cells; see Hirose *et al.*, 1991) in the subcuticular regions where the partial tunic fusion is occurring (Figs. 4–6); we are not sure which of these events occurs earlier. Many of the infiltrating blood cells are morula cells that have several vacuoles filled with electron-dense material. The disintegrating tunic cells discharge electron-dense materials that probably originate from their granules. The discharged materials seem to bind to the filamentous components of the tunic matrix and form electron-dense filaments around the cells (arrows in Fig. 4). Afterward, the infiltrating blood cells migrate around the regions of partial tunic fusion (Fig. 7), and then they disintegrate (Fig. 8). The disintegrating morula cells discharge electron-dense materials, as seen around disintegrating tunic cells, and electron-dense filamentous structures are also formed in the tunic (Fig. 8).

The infiltrating blood cells increase in number, and they disintegrate and discharge their contents in the tunic (Figs. 9 and 10). Electron-dense fibers appear around the disintegrating cells (arrows in Fig. 10). This inflammatory-like reaction occurs in a limited area, because the infiltrating cells usually disintegrate after migrating to a subcuticular location where the tunics have partially fused. The cell infiltration, migration, and disintegration proceed further, and eventually the migrating cells form an aggregate in those subcuticular regions where the tunics of the allogeneic colonies have partially fused (Figs. 11 and 12). Within the cell aggregates, disintegrating cells discharge their contents, *i.e.*, electron-dense, eosinophilic materials. Around them is some electron-dense substance that may be derived from the discharged materials.

Separation of the rejection lesion

When the rejection is complete, a dense continuous layer appears in the tunic matrix, and it separates both colonies from the rejection lesion (Figs. 13–16). This new wall consists of aggregates of electron-dense fibers (arrows in Figs. 14 and 16).

Discussion

When colonies are brought into contact at their growing edges, the first interaction is a dissolution of the cuticles of the contacting tunics. Due to the cuticle dissolution, the tunics of the two colonies undergo partial fusion. Even when rejection is induced by contact between cut surfaces, the tunic matrixes of the two colonies appear to fuse shortly after the contact (Hirose *et al.*, 1990b). The tunic matrices facing each other probably fuse without allorecognition. At this stage, morphological differences are not observed between compatible and incompatible combinations, but the signs of rejection soon appear. Allorecognition probably occurs shortly after the tunics partially fuse. Blood plasma induces the allospecific reaction in both *Botryllus primigenus* (Taneda and Watanabe, 1982) and *Botrylloides simodensis* (Saito and Watanabe, 1984). Probably, alloreactive factors diffuse into the opposite colony through sites of partial fusion, and the alloreactive cells respond to these factors. Furthermore, the allorecognition sites are

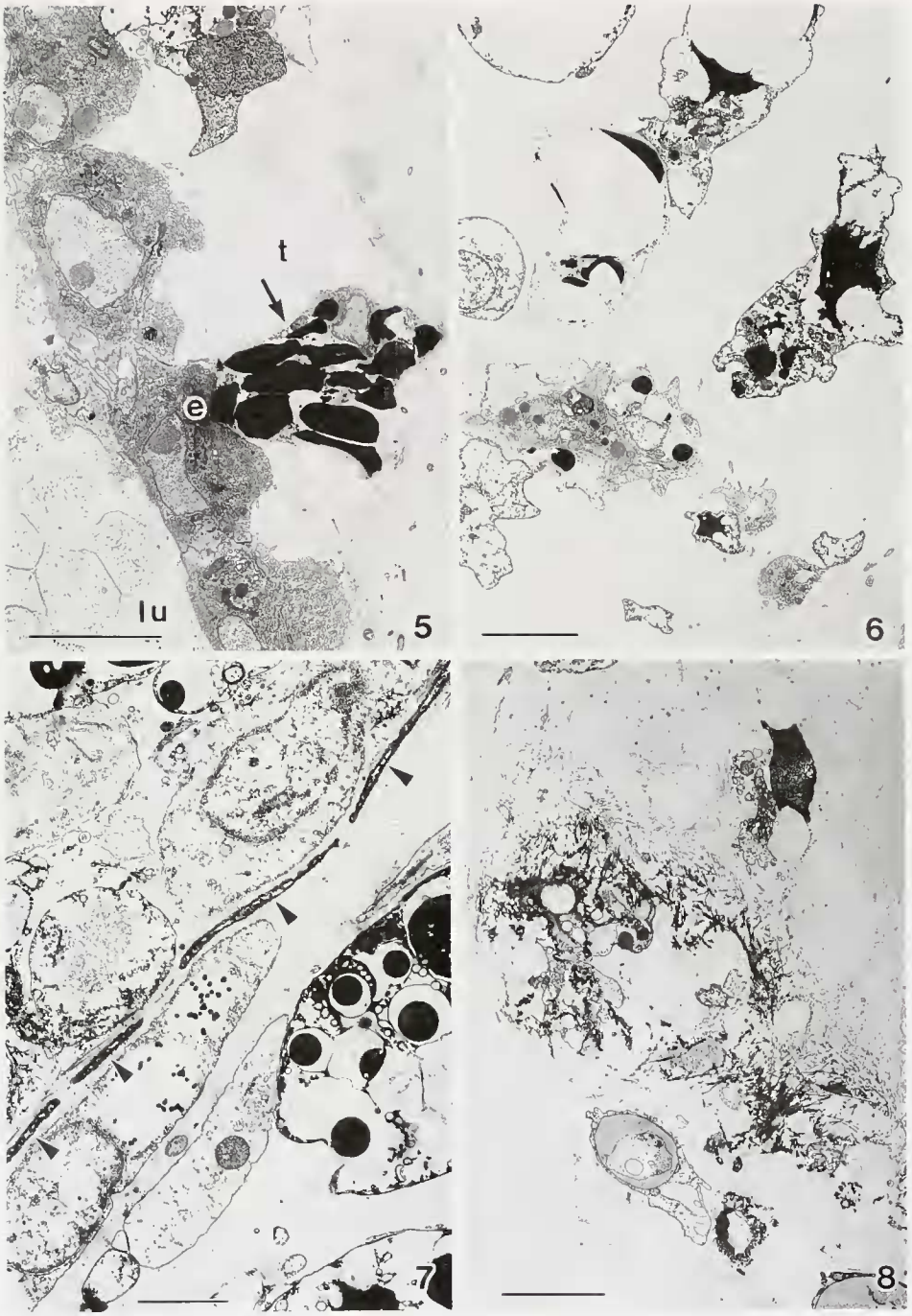


Figure 5. Blood cell infiltration (*Botrylloides simodensis*). Arrow, infiltrating blood cell (morula cell); e, epidermis of blood vessel; lu, vascular lumen; t, tunic matrix. Bar = 5 μm.

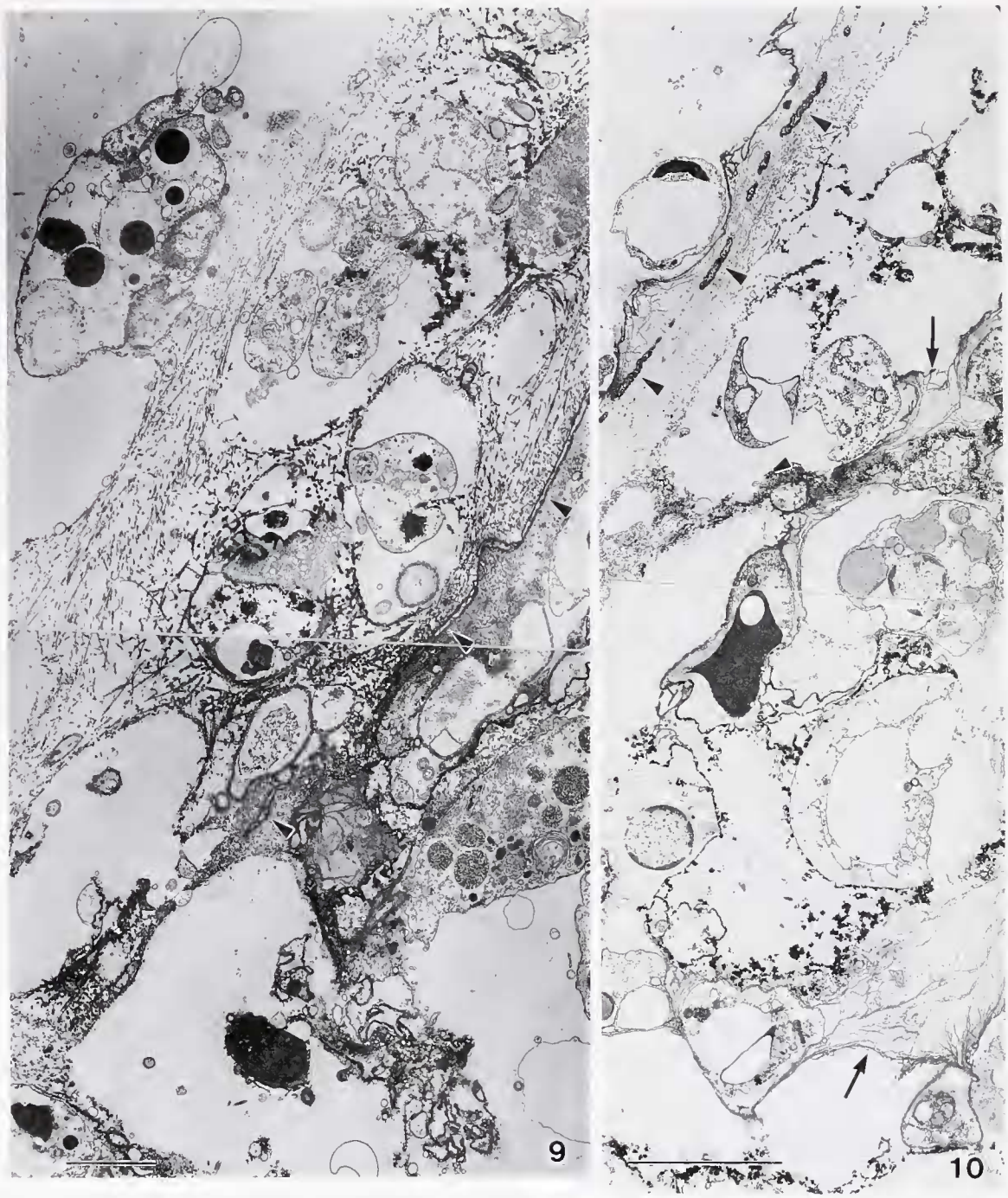
Figure 6. Blood cells infiltrating in the tunic (*Botrylloides fuscus*). Bar = 5 μm.

Figure 7. Blood cells migrate in an area of partial tunic fusion (*Botrylloides simodensis*). Arrowheads indicate tunic cuticle. Bar = 2 μm.

Figure 8. Disintegration of infiltrating blood cells (morula cells) in the tunic (*Botrylloides fuscus*). Electron-dense materials are discharged in the tunic. Bar = 5 μm.

probably restricted to the tunic, particularly in species in which surgical fusion occurs, *i.e., B. fuscus;* moreover, the tunic cells may well be the sole allorecognition sites. On the other hand, the ampullar epithelia or some kinds of blood cells may also be alloreactive in *B. simodensis,* because an intensive rejection occurs between incompatible colonies that make contact at artificial cut surfaces.

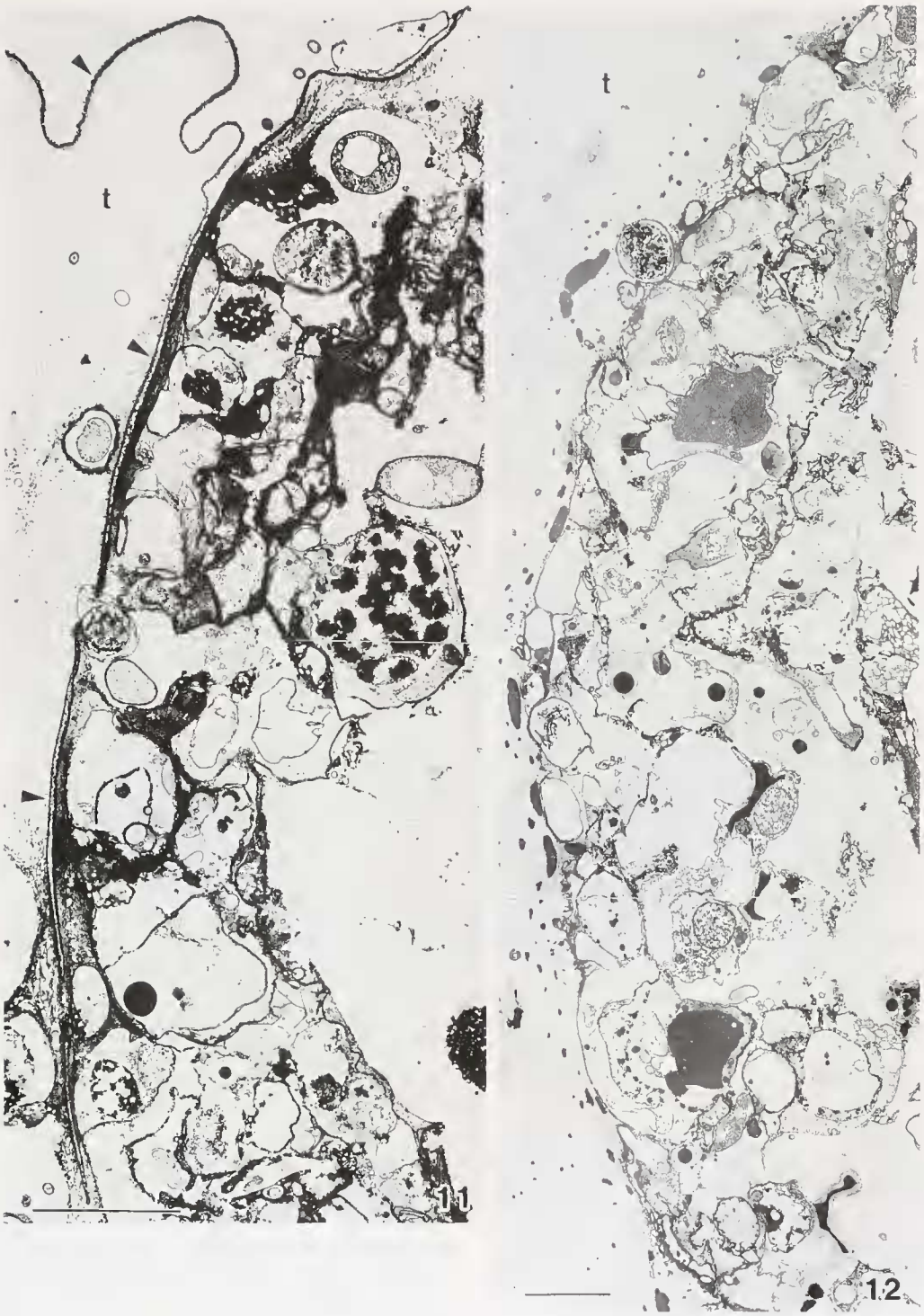
The first signs of SCR are the disintegration of vacuo-



Figures 9 and 10. Disintegration of blood cells in the vicinity of a partial fusion of tunic cuticles (Fig. 9, *Botryllodes simodensis*, Fig. 10, *B. fuscus*). Electron-dense fibers are formed in the tunic (arrows in Fig. 10). Arrowheads, tunic cuticle. Bar = 2 μm, Fig. 9; 5 μm, Fig. 10.

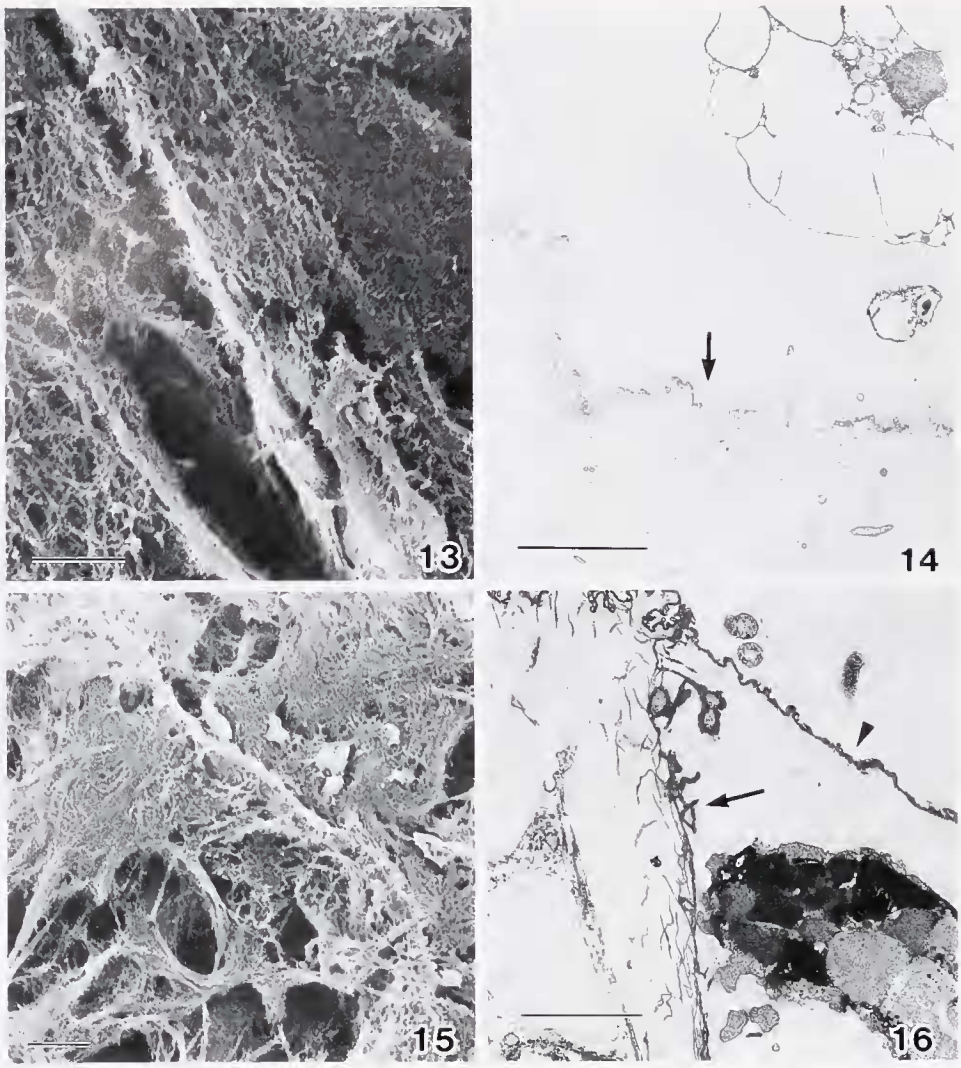
granular tunic cells and the infiltration of blood cells, mainly morula cells. The disintegrating tunic cells may release chemotactic factors that induce blood cell infiltration. Whether the tunic cell disintegration is a primary response to the allorecognition or an induced one remains uncertain. The infiltrating blood cells migrate and disintegrate at limited sites in the subcuticular region during SCR; the discharged contents from blood cells might also contain chemotactic factors that induce blood cell infiltration and disintegration.

The disintegrating tunic cells and morula cells discharge electron-dense materials, and these materials ap-



Figures 11 and 12. Cell aggregates at the rejection lesion (Fig. 11, *Botrylloides simodensis;* Fig. 12, *B. fuscus*). Arrowheads, tunic cuticle; t, tunic matrix. Bar = 5 μm.

pear to bind to the tunic matrix to form filamentous structures around the disintegrating cells. The discharged contents probably rearrange the tunic matrix and induce the disintegration of the tunic structures. The formation of filaments in the tunic around the disintegrating cells was reported in the allogeneic rejection of *Botryllus primigenus* (Tanaka, 1973; Tanaka and Watanabe, 1973). In some solitary ascidians, morula cells (or



Figures 13–16. New wall separating the colony from the rejection lesion (Figs. 13 and 14, *Botrylloides simodensis*, Figs. 15 and 16, *B. fuscus*). Figures 13 and 15 are scanning and Figures 14 and 16 are transmission electron micrographs. Lower left parts are lesion areas in these figures. Arrows, new wall; arrowheads, tunic cuticle. Bar = 5 μm, Figs. 13 and 14; 10 μm, Fig. 15; 2 μm, Fig. 16.

vacuolated cells) contain phenoloxidase, which has cytotoxic activity (Jackson *et al.*, 1993), and they release the enzyme in response to allogeneic blood cells (Akita and Hoshi, 1995). In *Botryllus schlosseri*, a botryllid ascidian, Ballarin *et al.* (1993) showed that the morula cells contain phenoloxidase, as well as peroxidase and arylsulfatase; and these authors also showed that phenoloxidase is involved in the allogeneic rejection reaction (Ballarin *et al.*, 1994, 1995). In the SCR of *Botrylloides* species, the discharged substances from morula cells also probably contain phenoloxidase.

In the SCR, the infiltrating blood cells form aggregates in the subcuticular region where partial fusion occurs. There may be a regulatory system to restrict the rejection reaction to the limited areas. In contrast, when rejection is induced by contact between cut surfaces in *B. simodensis*, the infiltrating cells disintegrate at random around the cut surface facing the incompatible colony, and the rejection lesion is large enough to be observed as a clear, black line (Hirose *et al.*, 1990b). In this case, the regulatory system proposed above would not work.

After the rejection reaction, the new wall appears, separating the rejection lesion from the colonies. The synthesis of the new wall was first described in the allogeneic rejection in *B. primigenus* (Tanaka, 1973; Tanaka and Watanabe, 1973), and the same structures were also reported in the rejection induced by the contact of allogeneic colonies of *B. simodensis* at cut surfaces (Hirose *et*

al., 1990b). Since the fine structure of the new wall is the same as that of regenerating tunic cuticle (Hirose *et al.*, 1995), the new wall should eventually become a tunic cuticle. During cuticle regeneration, the electron-dense fibers are probably produced from the aggregates of tunic matrix, and proteolysis is probably involved. In SCR, the disintegrating cells may release factors that induce the aggregation of tunic matrix and lead to the production of the electron-dense fibers. The new wall is supposed to act as (1) a barrier that prevents the migration of cells to the disintegrating tissue, (2) a barrier preventing the diffusion of factors into the colony from the allogeneic colony and disintegrating cells, (3) an outer covering of the tunic once the rejection lesion has been cleared.

What cell types in a colony discriminate self from nonself? The most likely candidates are the tunic cells, particularly in *B. fuscus* where surgical fusion occurs between incompatible colonies at the cut surface (Hirose *et al.*, 1994). In this species, surgical fusion indicates that the distribution of allorecognition sites is restricted to the tunic, and thus tunic cells may be the sole allorecognition sites. The morphological changes that occur during the fusion and rejection reactions also suggest that the allorecognition is carried out only in the subcuticular region of the tunic that is partially fused with the contacting colony.

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